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(57) Abstract

Oligodeoxynucleotides are rendered more resistant to degradation by nucleases by the inclusion of a base sequence at the 3' end which forms a hairpin loop-type secondary structure. The hairpin loop sequence forms an extremely stable structure comprised of a double stranded stem and loop region, 3' to the single stranded antisense sequence. The hairpin loop structures are used advantageously in stabilising antisense oligodeoxynucleotides which are used to block gene expression *in vivo* or *in vitro*.

JC4HP1	5' ACCATTTCGACATCTGGA	**GCGTTCGC** 3'
JC4HP2	5' ACCATTTCGACATCTGGA	**GCGAAAGC** 3'
JC4SHP2	5' GTACCTTGACTCAGTACA	**GCGAAAGC** 3'
JC4HP2S	5' ACCATTTCGACATCTGGA	**ACGAGCAG** 3'
AS3HP2	5' TGCATAAAATGTCTGC	**GCGAAAGC** 3'
JC4HP3	5' ACCATTTCGACATCTGGA	**GCGTAAGC** 3'

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STABLE OLIGONUCLEOTIDES

The invention relates to oligonucleotides and in particular to oligodeoxynucleotides which have been modified so that they are relatively resistant to degradation by nucleases. The invention has particular relevance to antisense oligodeoxynucleotides and the inhibition of gene expression both in vivo and in vitro.

Many disease states are the result of the expression of abnormal genes within cells. These may be human genes found mutated in many diverse premalignant and malignant lesions such as p53 which is a tumour suppressor gene. Foreign genes can become integrated into cellular DNA, for example after viral infection, and can subsequently be expressed by the cells. The expression of such genes provides obvious therapeutic targets, and over the past years the idea of using antisense oligonucleotides as agents to specifically inhibit expression of these genes has become popular. Other viral infections, where no integration of viral DNA occurs are also suitable targets, as the translation of essential viral genes in infected cells may be blocked. Other disease states may also present opportunities for the therapeutic use of antisense oligonucleotides, as they result from over expression of normal gene products.

The basic principle of antisense technology relies on the complementary base pairing of a short oligonucleotide of either RNA or DNA, with DNA or with RNA transcribed from the gene of interest. This pairing can prevent the protein product of the gene from being expressed. The base pairing can be chosen so that the antisense strand is complementary to a particular gene transcript.

Antisense mediated arrest of translation was first demonstrated in cell-free systems several years ago (Paterson, B.M. et al. (Proc. Natl. Acad. Sci. USA 74:4370-4374 (1977)); Hastie, N.D. and Held, W.A. (Proc. Natl. Acad. Sci. USA 74:1257-1271 (1978)); and later in eukaryotic cells (Izant,

J.G. and Weintraub, H. (Cell 36: 1007-1015 (1984)). The potential of the technique was not widely investigated until the automated chemical synthesis of oligonucleotides became possible.

In recent years the automated chemical synthesis of oligonucleotides of desired sequence has meant that antisense technology has been applied to different areas. The topic has been reviewed many times (Zon, G. (Pharmaceutical Res. 5 (9): 539-549 (1988); Van der Krol, A.R. et al. (Biotechniques 6: 958-976 (1988); Stein, C.A. and Cohen, J.S. (Important Advances in Oncology 79-97 Ed. De Vita, V.T. Lippinicott Press (1989); Helene, C. and Toulme, J.J. (BBA 1049: 99-125 (1990); Tidd, D.M. (Br. J. Cancer 63: 6-8 (1991)). Antisense oligonucleotides have been shown to be capable of modifying the expression of their target genes in a wide range of experimental systems. In several in vitro cell-free systems, such as rabbit reticulocyte lysate (Minshull, J. and Hunt, T. (Nucl. Acids Res. 14 (16): 6433-6451 (1986); Sartorius, C. and Franklin, R.M. (Nucl. Acids Res. 19 (7): 1613-1618 (1991) and Krebs-2 cell-free system (Miroshnichenko, N.A. et al. (FEBS 234 (1): 65-68 (1988)). Cellular systems include Xenopus oocytes (Dagle, J.M., et al. (Nucl. Acids Res. 18 (16): 4751-4757 (1990)), and many cultured cell lines, for example HL-60 (Holt, J.T., et al. (Mol. Cell. Biol. 8 (2): 963-973 (1988)), and T15, N-ras transformed NIH3T3 cells (Tidd, D.M., et al. (Anti-Cancer Drug Design 3: 117-127 (1988)). There are also a limited number of in vivo examples (Wickstrom, E. et al. (FASEB J. 5: A1443 (1991)). Antisense oligodeoxynucleotides (ODNs) ranging in length from 7 to 28 base pairs (bp) have been reported to be effective in test systems (Van der Krol, A.R. Biotechniques 6:958-976 (1988)). To be therapeutically useful, an ODN must be specific for its target sequence, and it has been calculated statistically that a 14mer is the minimum length required to obtain only one exact match within the transcripts from the human genome (Ts'o, P.O.P., et al.

(Biological Approaches to the Controlled Delivery of Drugs 507: Ann. N.Y. Acad. Sciences (1987)).

To date, antisense technology has had the greatest impact in horticulture where antisense RNA produced in transfected cells has been used to modify petal colouration in Petunia by targeting Chalcone synthetase (Van der Krol, A.R., et al. (Nature 333: 866-869 (1988)), and to control tomato ripening by targeting the rate-limiting enzyme in the biosynthetic pathway of ethylene (Oeller, P.W., et al. (Science 254: 437-439 (1991)).

One problem with antisense oligonucleotides is that they are not readily taken up by tissues and cells. Approaches to improving cellular uptake include conjugation to poly(L-lysine) (Leonetti, J.P. et al. Gene 72: 323-332 (1988)), and inclusion of oligonucleotides in liposomes (Loke, S.L. et al. Curr. Top. Microbiol. Immunol. 141: 282-289 (1988); Leonetti, J.P. et al. Proc. Natl. Acad. Sci. USA 87: 2448-2451 (1990)).

The most significant problem associated with the use of antisense oligonucleotides is that they are rapidly degraded by nuclease activity in serum and in cells. The predominant nuclease activity affecting antisense efficacy is 3'-5' directed exonuclease activity. The problem of degradation has been partially resolved by modifying the exposed phosphate backbone of oligonucleotides in order to alter nuclease recognition and therefore confer some measure of resistance to degradation. Backbone modifications include methylphosphonate ODNs, where an oxygen of the phosphate group is replaced by a $-CH_3$ group (Miller, P.S. et al. (Biochemistry 20 (7): 1874-1880 (1981); Tidd, D.M. and Warenius, H.M. (Br. J. Cancer 60: 343-350 (1989)), and phosphorothioate ODNs, where an oxygen is replaced by a sulphur (Stec, W.J. et al. (J. Am. Chem. Soc. 106: 6077-6079 (1984)); Matsukura, M., et al. (Proc. Natl. Acad. Sci. USA 84: 7706-7710 (1987)).

Modifications have also been made to the bases and these modifications include the use of α-anomeric oligonucleotides which are nuclease resistant, but actually hybridise to RNA

in a parallel rather than the usual antiparallel manner (Bertrand, J.R. et al. (Biochem. & Biophys. Res. Comm. 164 (1): 311-318 (1989)); Rayner, B. et al. ("Oligodeoxynucleotides, Antisense Inhibitors of Gene Expression" Ed. Cohen. J.S. Macmillan (1989)). A further example is the addition of a 3' anthraquinone pseudonucleoside, which improves the strength of hybridisation without reducing specificity and also confers protection against exonuclease degradation (Lin, K.Y. and Matteucci, M. (Nucl. Acids Res. 19 (11): 3111-3114 (1991)). Synthetic oligonucleotide analogues have the disadvantage of potential toxicity to cells.

The present invention is therefore directed to ways of stabilising oligonucleotides against nuclease degradation.

The present invention provides an open oligodeoxynucleotide for inhibiting or modifying gene expression in vivo or in vitro comprising an antisense sequence of nucleotide bases and further nucleotides at one or both ends of the antisense sequence, one or both of the said further nucleotide sequences being capable of forming a secondary structure conferring at least partial nuclease resistance on the oligodeoxynucleotide.

An "open" oligodeoxynucleotide may be single stranded or double stranded. However there will always be free 3' and 5' ends.

A "closed" oligodeoxynucleotide may be single or double stranded but there will not be any free ends. A "closed" oligodeoxynucleotide may be termed "ligated" in some references.

The term in vivo above is intended to cover living organisms, tissues and cells whereas in vitro is intended to cover cell extracts and cell free translation systems. The inhibition or modification of gene expression will usually involve duplex formation between the oligodeoxynucleotide and the RNA or DNA sequence of interest to give an inhibition or modulation of translation. Also possible is the inhibition or modulation of gene transcription by the formation of

triplexes comprising an oligonucleotide and a double stranded duplex of DNA (Yoon et al. Proc Naltl. Acad Sci USA 89:3840-3844(1992)). The formation of triple helices has the potential to alter gene transcription (Le Doan et al. Nucl. Acids Res. 15:7749-7760 (1987)). The formation of triple helices can occur at regulatory regions of genes, these regions being the targets for sequence-specific DNA binding proteins. The DNA binding proteins have the potential to activate or repress transcription from gene promoters. Triple helix formation at sequence motifs recognised by DNA binding proteins may allow modulation of gene expression in order to effect upregulation by inhibiting the binding of a repressor or downregulation by inhibiting the binding of an activating protein. (Maher III et al. Antisense Research and Development 1: 227-281 (1991)). Triple helices are formed by Hoogsteen-type hydrogen bonds between thymine and protonated cytosines with Watson-Crick A.T. and G.C. pairs respectively. An oligodeoxynucleotide in accordance with the invention can therefore be used to inhibit the binding of a DNA binding protein to its binding site on DNA.

Oligodeoxynucleotides in accordance with the invention also include so-called chimeric oligodeoxynucleotides in which some or all of the nucleotide bases or part of the nucleic acid back-bone are chemically modified for stability against nuclease degradation or for other desirable properties such as good cellular uptake. The modified bases may be present as a sequence or as isolated bases within a sequence. An oligodeoxynucleotide may be provided in which all the bases are chemically modified. The bases forming the secondary structure may also be modified. Chemically modified back-bones and bases include phosphorothioates, methylphosphonates and 3' anthraquinone pseudonucleosides.

Oligodeoxynucleotides in accordance with the invention may also be conjugated to poly(-L-Lysine) or incorporated in liposomes in order to improve their cellular uptake.

The invention includes an open oligodeoxynucleotide for use in the treatment of the human or animal body by therapy or diagnosis by inhibition of the expression of a gene product, the said oligodeoxynucleotide comprising an antisense sequence of nucleotide bases and further nucleotides at one or both ends of said antisense sequence one or both of the further nucleotide sequences being capable of forming a secondary structure conferring at least partial nuclease resistance on the oligodeoxynucleotide.

Preferably, there is one secondary structure present and it is positioned at the 3' end. Therefore the antisense oligonucleotide is resistant to 3'- 5' directed exonuclease attack.

The said further nucleotide sequence forming the secondary structure preferably comprises at least 5 nucleotide residues. Prefereably, the secondary structure comprises eight nudeotide residues. The secondary structure may comprise up to 100 nucleotide residues.

Preferably the secondary structure is a hairpin loop.

The hairpin loop structure has a portion where nucleotide bases are paired together and a portion where they are unpaired. The unpaired portion is generally kinked or looped back on itself. The paired portion forms a double stranded stem which supports the loop. The part of the sequence actually forming the loop may itself undergo non Watson-Crick type base pairing.

The said further nucleotide sequence forming the hairpin loop structure is preferably 5' GCGAAAGC 3'. This structure has been found to be particularly effective and is the most preferred structure.

The said further nucleotide sequence forming the hairpin loop structure may be 5' GCGTTCGC 3'.

The said further nucleotide sequence forming the hairpin loop structure may be 5' GCGTAAGC 3'.

The oligodeoxynucleotide preferably comprises more than 12 nucleotide residues.

The invention includes an open oligodeoxynucleotide comprising a sequence of more than twelve nucleotide residues and which has further nucleotide residues which form a secondary structure at one or both ends of the said sequence, the secondary structure conferring at least partial nuclease resistance on the oligodeoxynucleotide.

Preferably the oligodeoxynucleotide is capable of hybridising with at least a portion of a selected mRNA species.

The invention includes any oligodeoxynucleotide as herein-before defined in a pharmaceutically acceptable carrier. For example the oligodeoxynucleotide may be incorporated in liposomes. Such preparations include topical preparations e.g. ointments or creams where application of the oligodeoxynucleotide is to be made to the skin.

The oligodeoxynucleotides in accordance with the invention may include at least one chemically modified nucleotide base.

The oligodeoxynucleotides may have at least a portion of the sugar phosphate backbone modified chemically.

The invention also includes an oligodeoxynucleotide as herein defined for the manufacture of a medicament for use in a method of medical treatment of an organism, the said treatment involving blocking the expression of a selected gene or selected genes.

The stable oligodeoxynucleotides of the present invention are not restricted in their use to the treatment of human or animal diseases. They will of course be of widespread application wherever nuclease resistant oligodeoxynucleotides are required. For example the modification or inhibition of gene expression in plants and fungi, bacteria and other prokaryotes. Another area where the stable oligodeoxynucleotides of the invention will be of use is in diagnostics where crude cell extracts or extracelluar material could be screened using a labelled oligodeoxynucleotide probe which hybridises to a specific base sequence. The probe would

carry at least one stabilising secondary structure.

The invention will now be described in detail by way of Examples 1 to 9 and with reference to Figures 1 to 15 in which:

Figure 1 is an autoradiograph of the Northern blot described in Example 1.

Figure 2 shows the sequences and target sites of anti-sense oligodeoxynucleotides (ODNs) around an AUG start codon associated with the sequence for vaccinia virus DNA polymerase.

Figure 3 is the thermal denaturation curve used for determining T_m values in Example 1.

Figure 4 is an autoradiograph of the 15% polyacrylamide (PAGE) gel described in Example 1.

Figure 5 is a histogram representing the results of the experiments described in Example 2.

Figure 6 is a histogram representing the results of further experiments described in Example 2.

Figure 7 shows an autoradiograph of the 20% PAGE (7M urea) gel described in Example 3.

Figure 8 shows sequences of ODNs tagged with the 3' hairpin sequences described in Example 4.

Figure 9 shows autoradiographs of the 20% PAGE gels (7M urea) described in Example 4.

Figure 10 is an autoradiograph of the 20% PAGE (7M urea) gel described in Example 4.

Figure 11 shows an autoradiograph of the PAGE gel described in Example 5.

Figure 12 shows an autoradiograph of the PAGE gel described in Example 6.

Figure 13 is the graph used for determining T_m values described in Example 7.

Figure 14 is a histogram representing the results of the experiment described in Example 8.

Figure 15 comprises histograms representing the results

of the experiments described in Example 8.

The following Examples 1 to 3 set out background to the exemplification of the invention which follows in Examples 4 to 9.

EXAMPLE 1 (Not an example of the invention)
SYNTHESIS AND PROPERTIES OF ANTISENSE ODNs TO DNA POLYMERASE OF VACCINIA VIRUS

Vaccinia virus is a member of the poxviridiae family and is generally regarded as the prototype poxvirus. The virions are large (200-400 nm) and the 186 kb double stranded DNA genome is contained in a central core. The genome is characteristic of the poxviruses in that it is A/T rich, has inverted terminal repeats, covalently cross-linked ends and termini which exist as hairpin loops. During the life cycle, the virus is adsorbed on to cells before entering. Once inside a cell the virus particle uncoats and the early genes are transcribed. The viral DNA is then replicated prior to transcription of late genes. Mature virus particles, once assembled, migrate to the Golgi apparatus where they become enveloped in a double membrane before release from cells.

Vaccinia virus is easily grown in cell culture and so antisense ODNs can be tested against live virus in a cell culture system, allowing quantitation of the effect of antisense ODN on viral titre. The entire sequence of the Copenhagen strain of vaccinia is known (Goebel, S.J. et al, (Virology 179: 247-266 (1990)), and many sequences have been obtained for individual genes of other strains, such as WR. Vaccinia encodes several virus specific proteins which provide suitable antisense targets (Moss, B. ("Replication of Poxviruses in Virology"; 685-703 Fields, B.N. et al eds. Raven Press, New York (1985)). Examples are the DNA polymerase, RNA polymerase, and topioisomerases. Of these the DNA polymerase gene, which was sequenced in the WR strain several years ago (Earl, P.L. et al. (Proc. Natl. Acad. Sci. USA 83: 3659-3663 (1986)), is the most appropriate target due to its absolute

requirement for virus proliferation (Moss, B. and Cooper, N. (J. Virol. 43: 673-678 (1982)), and the fact that unlike the RNA polymerase the protein is not present in the virus particle at the start of the life cycle.

The 1.1 kb portion of the 5' end of the vaccinia virus WR strain was cloned and transcribed in vitro.

The 5' region of the WR vaccinia virus DNA polymerase gene was amplified by Polymerase Chain Reaction (PCR). An ATG triplet upstream of a target initiation codon is removed and a stop codon introduced in the resultant 1107 base pair fragment in order to enhance translation in a rabbit reticulocyte lysate. The PCR product was cloned into the transcription vector Bluescript (Stratagene) to produce the construct pBS-VACC(2). A 1.05 kb RNA corresponding to the cloned portion of the DNA polymerase coding region was transcribed in vitro as described below.

PBS-VACC(2) was linearised by overnight digestion with BamH1 to produce a template for the production of run off transcripts. Transcriptions were carried out using a mCAP mRNA capping kit (Stratagene), this includes a 5'7meGpppG5' cap analogue which is used to initiate transcription, the resultant RNA being more efficiently translated in an in vitro system. T3 RNA polymerase was used for transcription of the sense RNA.

The resultant RNA was 1.05 kb which was the expected length and this was detectable on a Northern blot using ^{32}P γ ATP end-labelled ODN (JC3 sequence shown in Figure 2) as a probe (see Figure 1).

The autoradiograph shows that RNA of the correct size is transcribed in vitro from the construct pBS-VACC(2). This is detected on a Northern blot using the antisense oligonucleotide JC3 as a probe, illustrating that the RNA does contain the relevant target sequence and that the oligonucleotide will bind to this under stringent conditions.

The various antisense ODNs and control ODNs used in these Examples were prepared by a standard method of oligonucleotide

synthesis, familiar to the average worker skilled in the art. ODNs were synthesised on an ABI 380B (Applied Biosystems) using standard techniques. ODNs were purified on NAP10 columns (Pharmacia) or by reverse phase chromatography on an OPC Column (Applied Biosystems). The purity of the ODNs was analysed by capillary electrophoresis.

The ODN sequences and target sites are shown in Figure 2. Characters which are underlined indicate the position where base changes have been made in the cloned sequence from those in the wild type sequences (shown), to those shown in the PCR primers VACC(1) and VACC(2). All the antisense ODNs are complementary to the initiation codon region (AUG) of the DNA polymerase gene. JC2 is a 14mer which spans a base change engineered into the cloned sequence to enhance translation. Therefore, JC2 (mod) was synthesised for use in the in vitro translation assay. JC3 is an 18mer ODN which avoids this mismatch. JC4 is a second 18mer complementary to a region of RNA lying nine bases upstream of the JC3 target site, encompassing the JC2 target site.

Binding kinetics for the hybridisation of the antisense ODNs to the in vitro transcript were determined by quantitation of ^{32}P end-labelled ODN released from the target RNA with increasing temperature. T_m values, that is the temperature at which 50% of the oligonucleotide has dissociated from the RNA, were determined as follows.

20 pmoles of oligonucleotide were end-labelled using T4 polynucleotide kinase and $\gamma^{32}P$ ATP. The in vitro transcribed RNA was diluted to - 100 ng/µl in RNA denaturing buffer (20% (v/v) formaldehyde, 6xSSC) and denatured by 10 min incubation at 65°C, freezing at -20°C overnight, followed by a second 65°C incubation. 100ng of denatured RNA was fixed to 0.5 cm diameter discs of Hybond-N (Amersham) by 15 seconds UV illumination and 2 hours baking at 80°C.

Discs were prehybridised for 2 hrs at 65°C in 2 ml of ODN prehybridisation solution (6xSSC, 0.01 M Sodium phosphate pH 6.8, 1mM EDTA pH8.0, 0.5% (w/v) SDS, 100mg/ml denatured

fragmented salmon sperm DNA, 0.1% (w/v) Nonfat dried milk). The labelled ODN was then added to the prehybridisation solution to give a concentration of 5 pmol/ml. Hybridisation was 2 hours at 65°C, cooling slowly overnight to room temperature for annealing of ODNs to the RNA.

Discs were washed 3 times in 3x SSC or a near physiological buffer ((PB)=0.1 M NaCl, 1 mM EDTA, 10 mM sodium phosphate pH 7.2) and counted to check hybridisation. Discs were then incubated in 1 ml of 3x SSC or PB for 5 mins at the start temperature, the 3x SSC or PB was removed to a scintillation vial and the disc washed in a further 1 ml of 3x SSC or PB which was pooled with the former. This step was repeated at increasing temperature, using temperature intervals of 2.5 or 5°C. A final wash was conducted at 100°C to remove all counts from the discs. The disintegrations per minute for each wash were determined by Cerenkov counting for 60 sec in a LKB counter. The percentage of the total counts released at each temperature was then plotted and used to determine the temperature at which 50% of the labelled ODN has dissociated from the bound RNA; that is the T_m value.

Figure 3 shows that the T_m values for the hybridisation of the ODNs to the target RNA in 3xSSC are 41°C for JC2, 57°C for JC3 and 53°C for JC4.

The RNA produced <u>in vitro</u> was translated into its encoded proteins in a rabbit reticulocyte lysate. SDS PAGE analysis of translation products showed a major protein band of about 40KD.

The ability of the antisense ODNs to inhibit <u>in vitro</u> translation was tested in relation to control ODNs by incubation of ODNs with RNA prior to addition of the lysate. 50 ng of the <u>in vitro</u> transcript was used in each <u>in vitro</u> translation reaction. Translation reactions were carried out using rabbit reticulocyte lysate (GIBCO-BRL). 10 µl reaction volumes comprised by volume 33% lysate, 8% 1M potassium acetate (pH 7.2), 10% translation mixture (- methionine, GIBCO-BRL) in the presence of ^{35}S methionine (Amersham). In

uninhibited reactions the in vitro RNA was made up to 5 µl with diethylpyrocarbonate (DEPC) treated H_2O, before addition of the components listed above. For translations conducted in the presence of antisense or control ODNs, the RNA and ODN were mixed at room temperature prior to addition of the translation reagents. Reactions were incubated at 30°C for 1 hour. Translation products were quantitated by TCA precipitation of synthesised proteins and scintillation counting. The products were analysed by electrophoresis on denaturing 15% SDS-PAGE gels.

Figure 4 shows analysis of in vitro translation products from various reactions on a 15% SDS PAGE gel. Lanes 1 to 11 are as follows; Lane 1 = blank sample containing no exogenous RNA, Lane 2 = The translation of 50 ng of the in vitro RNA transcript, Lanes 3 to 11 all contained 50 ng RNA in the presence of various ODN's, Lane 3 = + 75µmJC2, Lane 4 = + 25µmJC3, Lane 5 = + 50µmJC3, Lane 6 = + 25µmJC4, Lane 7 = + 50µmJC4, Lane 8 = + 25µmJC4HP1, Lane 9 = + 50µmJC4HP1, Lane 10 = + 75µmJC2 sense, Lane 11 = + 75µmJC2 nonsense.

The results show that JC2 prevents translation at 75µM. At lower concentrations JC2 is ineffective (data not shown). The longer antisense ODNs however, show effects at 25µM. In the case of JC4 total inhibition is seen at this concentration, whereas 50µM JC3 is required to obtain the same result. 75µM control sense and nonsense 14mer ODNs do not reduce translation in this system. These results show that expression of target RNA can be specifically inhibited in a cell free situation by the various antisense ODNs.

EXAMPLE 2 (Not an example of the invention)

THE EFFECT OF ANTISENSE ODNs ON VIRAL REPLICATION IN CELL CULTURE

The antisense ODNs were used against vaccinia virus infected confluent monkey kidney cells (CV-1).

Tests were carried out in 24 well plates (data not shown). The CV-1s were infected at a multiplicity of 0.0001

plaque forming units per cell (pfu/ cell). Controls were set up in which the cells were infected with the virus without the addition of antisense ODNs. After 48 hours the number of plaques formed in the controls (no antisense ODNs) was 27 (n = 2). The well with 25μM JC3 had 16 plaques. More significantly the plaques produced in the presence of the antisense ODN JC3 were visibly smaller indicating a reduced virus yield.

Further experiments were undertaken in 96 well plates to reduce the amount of ODN used. CV-1 cells were infected with vaccinia virus and then oligonucleotides were added. The vaccinia virus was harvested from these cells after 48 hrs and titred in fresh CV-1 cells to determine the total yield of virus. Results for CV-1s infected at a multiplicity of 0.1 pfu/cell are shown in Figure 5. Error bars represent replicate determinations of the harvested virus titre, n=6, 4 and 2 for the control, JC2 (mod) and JC3 respectively. The results for CV-1s infected at a multiplicity of 1pfu/cell are shown in Figure 6. In the presence of 18μM JC3 virus yield was reduced to 44% of the control values at an infectivity of 0.1 pfu/cell and 56% at an infectivity of 1 pfu/cell. 25μM of the 14mer ODN JC2(mod) which has a one base mismatch to the native target sequence, and JC2 nonsense which has no complementarity, resulted in no reduction of virus yield.

EXAMPLE 3 (Not an example of the invention)

THE EFFECT OF EXONUCLEASE ACTIVITY ON ANTISENSE ODNs

Two major problems arise in a cell culture system which are not encountered in a rabbit reticulocyte lysate. These are the uptake of the ODNs into cells and the degradation of the ODNs both in cell culture medium and within the cells. A 3' exonuclease is the predominant activity in both foetal calf serum (FCS) and the cell cytoplasm (Wickstrom, E. (J. Biochem & Biophys. Methods 1: 97-102 (1986); Boizau, C. & Toulme, J.J. (Biochimie 73: 1403-1408 (1991)). The effect of this activity on the vaccinia virus antisense ODNs was

investigated. 5' ^{32}P end-labelled JC3 was incubated in either 2.5% or 10% FCS in minimal essential medium (MEM) at 37°C. The control was MEM without FCS. Incubation was for up to 20 hr. Samples taken were subjected to on electrophresis on a 20% polyacrylamide in 7M urea gel. Figure 7 shows an autoradiograph of the gel. Lanes 1 to 4 show samples taken from serum-free MEM culture medium at t=0, 30 min, 1 hr and 2 hr. Lanes 5 to 9 show samples taken from MEM +2.5% FCS at t=0, 30 min, 1 hr, 2 hr and 20 hr. Lanes 10 to 14 show samples taken from MEM + 10% FCS at t=0, 30 min, 1 hr, 2 hr and 20 hr. ODNs in MEM showed no degradation. In 2.5% FCS there was significant degradation of the ODN indicated by the "ladder" of smaller molecular weight components and the depletion of the starting molecular weight band. At 10% FCS there was significant degradation after only 2 hr, equivalent to that seen after 20 hr in 2.5% FCS. These results indicate that 3' degradation of unmodified oligo-nucleotides is a limiting factor in the efficacy of antisense oligonucleotides in cell culture or <u>in vivo</u>.

<u>EXAMPLE 4</u>

<u>STABILISING STRUCTURES ATTACHED TO ODNs</u>

The ODNs shown in Figure 8 were synthesised using a standard method of oligonucleotide synthesis. The bases which are underlined form hairpin loop type secondary structures. Two different structures were used being designated HP1 and HP2. These were synthesised as part of the antisense sequence JC4 shown in Figure 2. The sequence JC4SHP2 is a control wherein the sequence of the antisense ODN is scrambled. JC4HP2S is also a control wherein the HP2 sequence is scrambled and so should form no secondary structure. The AS3HP2 sequence is an antisense ODN to the E6 gene of Human Papilloma Virus HPV16 including the HP2 hairpin loop.

The sequence JC4HP3 was also made. This comprises JC4 antisense sequence and the hairpin loop type secondary structure HP3. HP3 differs from HP2 in that there are two

adenosine residues and one thymidine residue, rather than three adenosine residues.

Figure 9 is a 20% polyacrylamide 7M urea gel showing analysis of ODN degradation products produced from 5' end-labelled antisense ODNs, JC4 and JC4HP1 in MEM+10% FCS at 37°C. Lanes 1 to 11 show samples taken at t=0, 30 mins, 1, 2, 3, 4, 5, 6, 7, 8 and 23 hr respectively. The gel shows that the 3' GC clamp structure of JC4HP1 prolongs the life of the ODN in 10% FCS with no degradation being seen until lane 5, i.e. there is little degradation for up to 3 hours.

Figure 10 shows a 20% polyacylamide 7M urea gel analysis of ODN degradation products produced from 5' end-labelled antisense ODNs targetted to HPV16 E6, (HPVAS3 and HPVAS3HP2) in MEM + 10% foetal calf serum at 37°C. Lanes 1 to 7 show samples of HPVAS3 taken at t=30 min, 1, 2, 3, 4, 5 and 19 hr. Lanes 8 to 14 show samples of HPVAS3HP2 taken at t=30 min, 1, 2, 3, 4, 5 and 19 hr respectively. The gel shows that HPVAS3HP2 is stable in 10% FCS for up to 19 hours. There are no apparent products of 3' exonuclease activity meaning that the HP2 structure is unexpectedly more stable than HP1. HP2 shows a many-fold greater stability than the unmodified ODNs.

EXAMPLE 5

THE EFFECT OF EXONUCLEASE ENZYME ON ODNs WITH OR WITHOUT A HAIRPIN LOOP STRUCTURE

The predominant nuclease activity in serum against single-stranded oligonucleotides is 3'-5' directed exonuclease (Abramova et al. Mol. Biol. Mosk 25(3):624-632 (1991)). In order to complement serum assays to ascertain the extent of degradation of the oligonucleotides, experiments were conducted to expose both native and hairpin-modified oligonucleotides (HPVAS3 and HPVAS3HP2 - sequences shown in Figure 8) to Phosphodiesterase I (from Crotalus adamanteus venom (Sigma)). Phosphodiesterase I releases 5' mononucleo-

tides from the 3' end of oligonucleotides (Hayashi, O. TIBS 1:9(1976)). The enzyme is therefore a 3'-5' exonuclease. The Phosphodiesterase I reactions were conducted as follows. The following components were added to make a final reaction volume of 10µl; 10X Reaction Buffer (0.33M Tris-acetate, 0.66M Potassium acetate, 0.1M Magnesium acetate), 0.5ng ^{32}P end-labelled DNA oligodeoxynucleotide, 100 ng unlabelled DNA oligodeoxynucleotide as a competitor, and 0.5ng-5ng of Phosphodiesterase I. The components were mixed and samples incubated in a water bath at 37°C. The reactions were visualised by electrophoresis on a 7M Urea 20% polyacrylamide gel followed by autoradio-graphy of the wet gel. Oligonucleotides HPVAS3 and HPVAS3HP2 were incubated in the presence of 0.5 ng and 5 ng of Phosphodiesterase I, each reaction was incubated at 37°C for 5, 10 and 15 minutes. In Figure 11 Lane 1; control reaction, the HPVAS3 oligonucleotide with no added enzyme. Lanes 2-4; 0.5ng of added Phosphodiesterase I for 5, 10 and 15 minutes respectively. Lanes 5-7; 5ng of enzyme for the same conditions. Lane 8; control reaction, the HPVAS3HP2 oligo with no added enzyme. Lanes 9-11 0.5ng and lanes 12-14 5ng of added enzyme for the above stated time intervals clearly show HPVAS3 as a degraded ladder of smaller oligonucleotides. Lanes 12-14 show that HPVAS3HP2 is clearly resistant to 3'-5' attack by Phosphodiesterase I.

EXAMPLE 6

THE EFFECT OF KLENOW FRAGMENT ENZYME ON ODNs WITH OR WITHOUT A HAIRPIN LOOP STRUCTURE

To understand how the hairpin structure and its inherent stability is vital to confer nuclease resistance, both normal and hairpin modified oligodeoxynucleotides were incubated in the presence of Klenow Fragment, derived from DNA Polymerase I of E. coli (Boehringher Mannheim) and free nucleotides. (The fragment also contains a 3'-5' exonuclease, active against both double and single-stranded DNA). The presence

of the 3' hairpin loop structure on a single-stranded oligodeoxynucleotide will provide a free 3' hydroxy end and a single-stranded template to prime DNA polymerisation across the molecule by the Klenow enzyme. The experiment is therefore an assay for the presence of a 3' hairpin loop on an oligodeoxynucleotide. The oligodeoxynucleotides compared were JC4HP2 and JC4HP2S. 20 µl final reaction volumes contained 10X Klenow buffer, 2 µl 0.5mM dNTP's, 0.5ng kinase labelled DNA oligonucleotide and 1 unit of Klenow enzyme. The void reaction volume was made up with deionised, distilled water. The reactions were incubated for various times between 5 and 15 minutes, and stopped by the addition of stop-mix and loaded on to a (7M Urea) 20% polyacylamide gel. In Figure 12 Lane 1; is the control reaction; JC4HP2 + no Klenow enzyme. Lanes 2-4 = increasing time of incubation with Klenow enzyme with JC4HP2, 5, 10, 15 minutes respectively. Lane 5 = control reaction, no Klenow enzyme added to JC4HP2S. Lane 6, incubation of JC4HP2S with Klenow enzyme for 15 minutes. A higher molecular weight band is observed in tracks 2-4 where JC4HP2 is incubated with Klenow enzyme. Presumably this band is the result of Klenow enzyme forming a double-stranded oligodeoxynucleotide from the single-stranded hairpin loop intermediate form. In contrast, the scrambled hairpin sequence oligodeoxynucleotide JC4HP2S in lane 6 does not show any double-stranded forms. Upon exposure to Phosphodiesterase I JC4HP2 is found to be resistant whereas JC4HP2S is degraded. Thus, the nuclease resistance of sequence modified oligodeoxynucleotides would appear to be due primarily to the presence of the hairpin loop structure.

EXAMPLE 7

PROPERTIES OF THE ODNs WITH STABILISING HAIRPIN LOOP STRUCTURES

The sequence of the hairpin loop used in these examples was carefully chosen in order to fulfil two major requirements; it must form a stable hairpin at 37°C, and it must be

compact enough, not to interfere with hybridisation between the oligonucleotide and its target sequence. It also should not compromise the entry of oligonucleotides into the cell. The sequence of 5'GCGAAAGC3' has unusual characteristics in that it forms an extra-ordinarily stable secondary structure in the form of a hairpin loop. Significantly, its melting temperature (the temperature where half of the oligonucleotide is found in the random coil conformation and half in the hairpin conformation) is 76.5°C under normal conditions and in 7M Urea, 53°C. One would expect that at 37°C the bulk of the oligonucleotide containing the sequence dGCGAAAGC would be adopting the structure of a hairpin loop. Consequently, sequence modified oligonucleotides were chemically synthesised in the 3'-5' direction, incorporating the hairpin loop at the 3' end of a defined single-stranded region. These oligonucleotides, prior to undergoing incubation in serum or with enzymes are heated to 95°C for 15 minutes and allowed to cool to room temperature (Xodo et al. Biochemistry 27:6321-6326 (1988)). This procedure firstly forces all oligonucleotides into the random coil conformation. Upon cooling, the oligonucleotides undergo a transition which is directed by their melting temperature to the hairpin state. The hairpin loop containing oligonucleotides can be stored for long periods at -20°C. The heating and cooling of the hairpin oligonucleotides before use helps to ensure that all the hairpin sequences are in the required conformation and hence helps to stabilise their resistance to nuclease attack.

The presence of hairpin loops does not sterically hinder hybridisation of the antisense ODN to its target RNAs. This is shown firstly by the T_m values for these hybridisations, and secondly by the fact that the ODNs can prevent translation of the target RNA in a rabbit reticulocyte lysate.

T_m determinations were conducted in a near physiological buffer. T_m determinations for JC4, JC4HP1 and JC4HP2 are shown in Figure 13. In the figure, (a) and (b) are two replicates of the same experiment. The graph shows the

dissociation of ^{32}P end-labelled ODNs: JC4, JC4HP1 and JC4HP2 from target RNA with temperature in a near physiological buffer. The released radiolabelled ODN was determined by Cerenkov counting at 2.5°C temperature intervals. JC4HP2 shows a release profile almost identical to that of the normal ODN, JC4, indicating that the modification has had little effect on hybridisation to the target RNA. JC4HP1 however leads to a 6°C increase in the determined T_m, but this may be due to "breathing" of this hairpin structure allowing some nonspecific binding of the ODN 3' extension to the RNA.

JC4HP1 prevents translation of RNA coding for the 5' end of the vaccinia virus DNA polymerase in rabbit reticulocyte lysate, see Figure 4, lanes 8 and 9. AT 25μM JC4HP1 does not completely prevent translation as seen with JC4, but inhibition is total at 50μM. Similar results are seen with JC4HP2 - data not shown.

EXAMPLE 8

THE EFFECT OF HAIRPIN LOOP STABILISED ODNs ON VIRAL REPLICATION IN CELL CULTURES

The hairpin ODNs were tested in the cell culture system described in Example 2. The effective concentration of ODN used was 25 μM. CV-1s were infected at a specific viral infectivity of 0.0005 pfu/cell and the virus yield after 48 hours determined. In Figure 14 the error bars indicate one standard deviation of the triplicate titres. At this infectivity, yields of vaccinia virus were reduced to 61%, 81% and 45% of the control value for JC3, JC4 and JC4HP1 respectively. At an infectivity of 0.0005 pfu/cell the addition of the HP1 structure appears to enhance the action of JC4, presumably by increasing the half life of the ODN.

Tests of JC4HP2 on vaccinia virus in cell culture were also carried out using a specific viral infectivity of 0.0005 pfu/cell and an effective concentration of 25 μM ODN. In Figure 15 the yield of vaccinia virus from CV-1 cells is shown for 24 hr and 48 hr post-infection. (a) is extracelluar virus

harvested from the cell culture medium, and (b) is intracellular virus harvested from the cells. Error bars indicate the standard deviation of triplicate titres. A reduced virus yield was seen in intracellular virus at 24 hours and in both extracelluar and intracellular virus at 48 hours, in the presence of 25μM JC4HP2 when compared to the ODN free controls. These reductions are 5, 2.7 and 3.2 fold respectively (i.e. 20%, 37% and 31% of the controls). From these preliminary results the HP2 modification therefore appears to enhance the antisense effect of JC4 in this system.

EXAMPLE 9

ANTISENSE OLIGONUCLEOTIDES AS ANTIVIRAL AGENTS AGAINST THE HUMAN PAPILLOMA VIRUS HPV16

Oligonucleotides having nucleic acid base sequences antisense to parts of the E6 or E7 genes of human papilloma virus (HPV16) are provided by a standard method of oligonucleotide synthesis. In addition to the antisense sequence, each oligonucleotide has at its 3' end a hairpin loop forming sequence. The hairpin loop forming sequence is one of 5'GCGTTCGC3', 5'GCGAAAGC3' or 5'GCGTAAGC3'.

The hairpin loop forming sequence renders the oligonucleotides antisense to E6 or E7 more resistent to nuclease degradation. The respective antisense oligonucleotides are able to reduce the expression of E6 and E7 genes in a cell free translation system. The stabilised antisense oligonucleotides should be capable of reducing the expression of HPV16 genes integrated into the genomes of SiHa or CaSki human cell culture lines.

CLAIMS

1. An open oligodeoxynucleotide for inhibiting or modifying gene expression in vivo or in vitro comprising an antisense sequence of nucleotide bases and further nucleotides at one or both ends of the antisense sequence one or both of the said further nucleotide sequences being capable of forming a secondary structure conferring at least partial nuclease resistance on the oligodeoxynucleotide.

2. An open DNA oligodeoxynucleotide for use in the treatment of the human or animal body by therapy or diagnosis by the inhibition or modification of the production of a gene product, the said oligodeoxynucleotide comprising an antisense sequence of nucleotide bases and further nucleotides at one or both ends of the oligodeoxynucleotide, one or both of the further nucleotide sequences being capable of forming a secondary structure conferring at least partial nuclease resistance on the oligodeoxynucleotide.

3. An oligodeoxynucleotide as claimed in Claim 1 or Claim 2, wherein a said secondary structure is at the 3' end.

4. An oligodeoxynucleotide as claimed in any one of Claims 1 to 3, wherein the said further nucleotide sequence forming the secondary loop structure, comprises at least 5 nucleotide residues.

5. An oligodeoxynucleotide as claimed in Claim 3 or Claim 4 wherein the said secondary structure is a hairpin loop.

6. An oligodeoxynucleotide as claimed in Claim 5 , wherein the said further nucleotide sequence forming the hairpin loop structure is 5' GCGAAAGC 3'.

7. An oligodeoxynucleotide as claimed in Claim 5, wherein the said further nucleotide sequence forming the hairpin loop structure is 5' GCGTTCGC 3'.

8. An oligodeoxynucleotide as claimed in Claim 5, wherein the said further nucleotide sequence forming the hairpin loop structure is 5' GCGTAAGC 3'.

9. An oligodeoxynucleotide as claimed in any preceding claim

comprising more than 12 nucleotide residues.

10. An open DNA oligodeoxynucleotide of more than twelve nucleotide residues including further nucleotide residues which form a secondary structure at one or both ends of the oligodeoxynucleotide conferring at least partial nuclease resistance on the oligodeoxynucleotide.

11. An oligodeoxynucleotide as claimed in Claim 10, which is capable of hybridising with at least a portion of a selected mRNA species.

12. An open oligodeoxynucleotide having a base sequence identical with any of the sequences shown in Figure 8.

13. An oligodeoxynucleotide as claimed in any one of Claims 1 to 12, wherein at least one of the nucleotide bases is chemically modified.

14. An oligodeoxynucleotide as claimed in any one of Claims 1 to 12, wherein at least a portion of the sugar-phosphate backbone is chemically modified.

15. An oligodeoxynucleotide as claimed in any preceding claim in a pharmaceutically acceptable carrier.

16. The use of an oligodeoxynucleotide as claimed in any one of Claims 1 to 14, for the manufacture of a medicament for the medical treatment of an organism, the said treatment involving the modification or inhibition of gene expression.

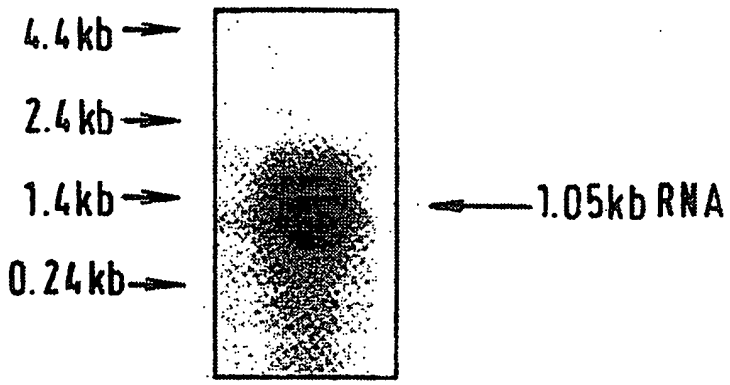

4.4kb
2.4kb
1.4kb
0.24kb
1.05kb RNA

FIG.1

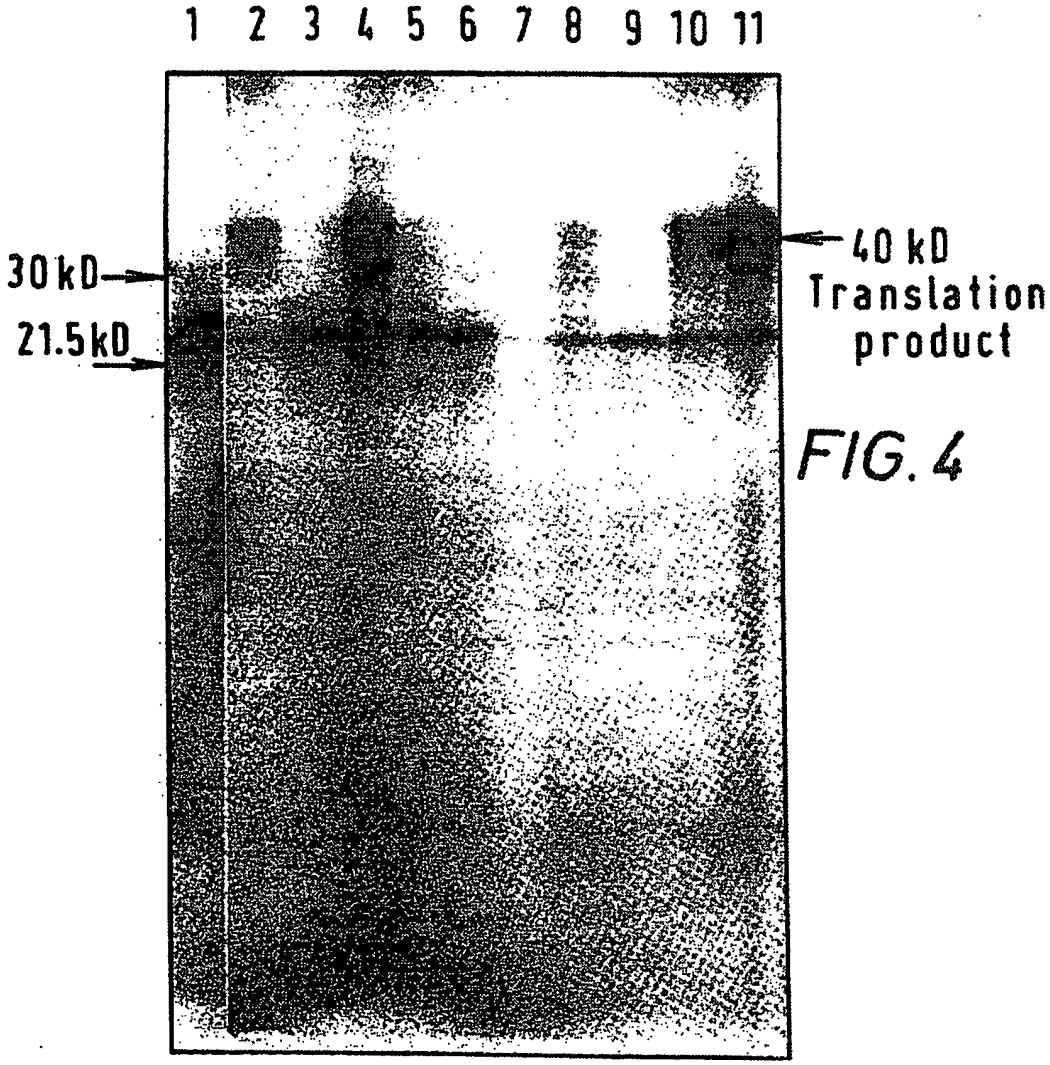

1 2 3 4 5 6 7 8 9 10 11
30kD
21.5kD
40 kD
Translation
product

FIG. 4

FIG.2

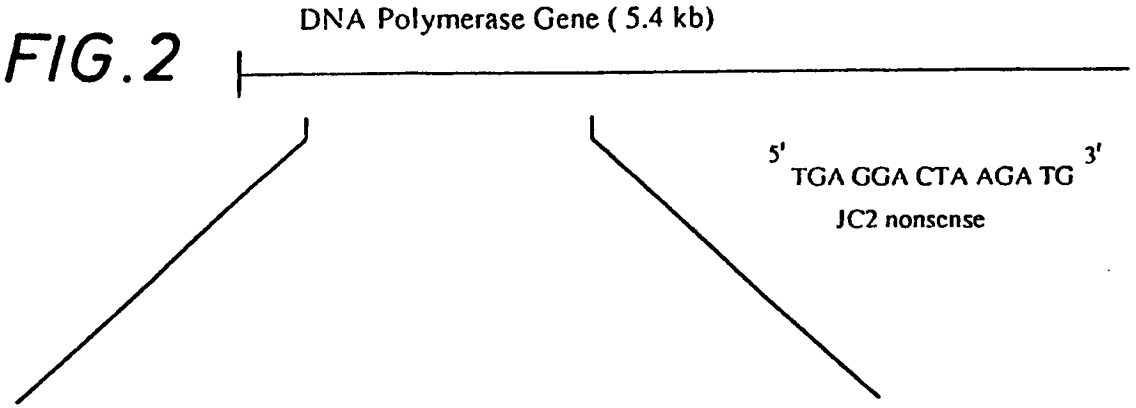

DNA Polymerase Gene (5.4 kb)
5' TGA GGA CTA AGA TG 3'
JC2 nonsense
5' A GAT GTC GAA ATG G 3'
JC2 sense
5' AT ATC TGA ATT CCA GGT GTC GAA ATG G 3'
VACC(1)
5'
3'
--TTT TAT ATC TGA AAT CCA GAT GTC GAA ATG GTG AGA CCG TAT TTA--
3' T CTA CAG CTT TAC C 5'
JC2
3' T CCA CAG CTT TAC C 5'
JC2(mod)
3' G CTT TAC CAC TCT GGC AT 5'
JC3
3' A GGT CTA CAG CTT TAC CA 5'
JC4
3' GCT TTC CGA TTC GGC GAC GTC AAC GTT 5'
VACC(2) Reverse PCR primer
Antisense to a region 1.1 kb downstream.

FIG. 3

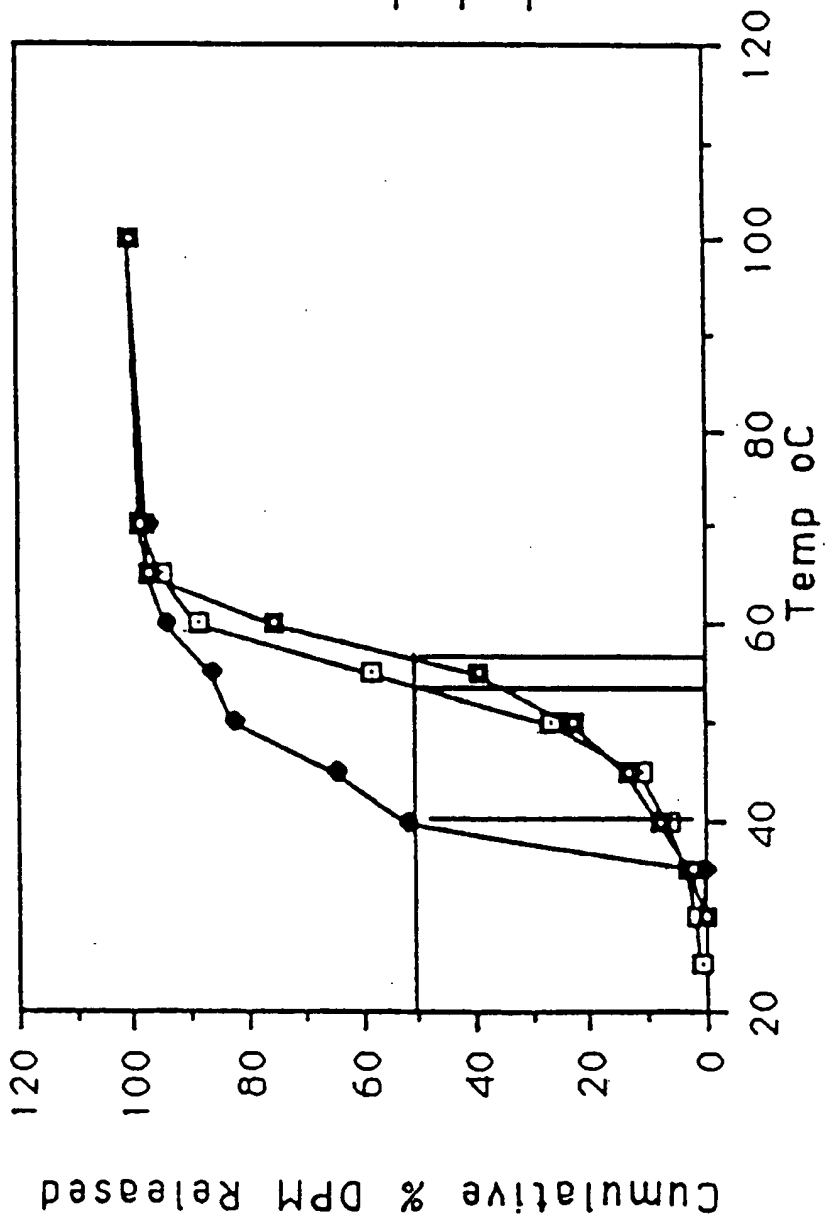

% JC4a
JC2(mod)
JC3
Cumulative % DPM Released
0
20
40
60
80
100
120
Temp oC
20
40
60
80
100
120

FIG.5

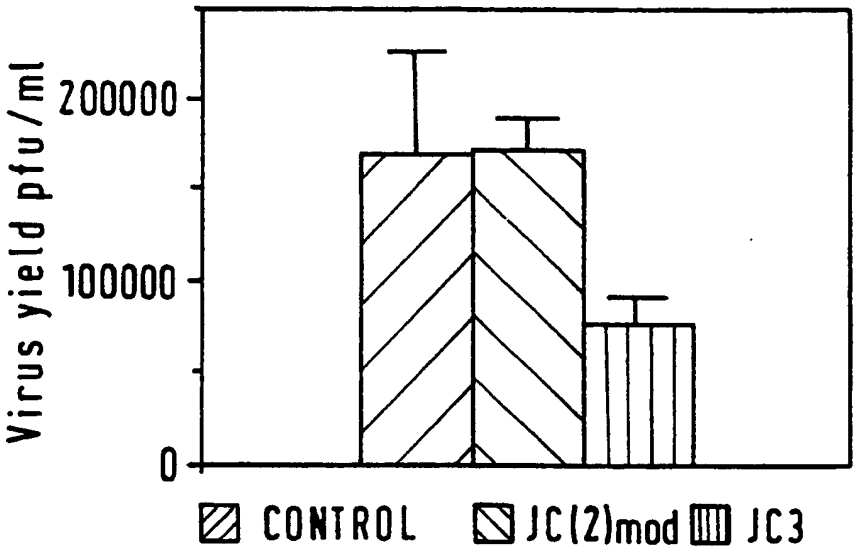

Virus yield pfu/ml
200000
100000
0
CONTROL
JC(2)mod
JC3

FIG.6

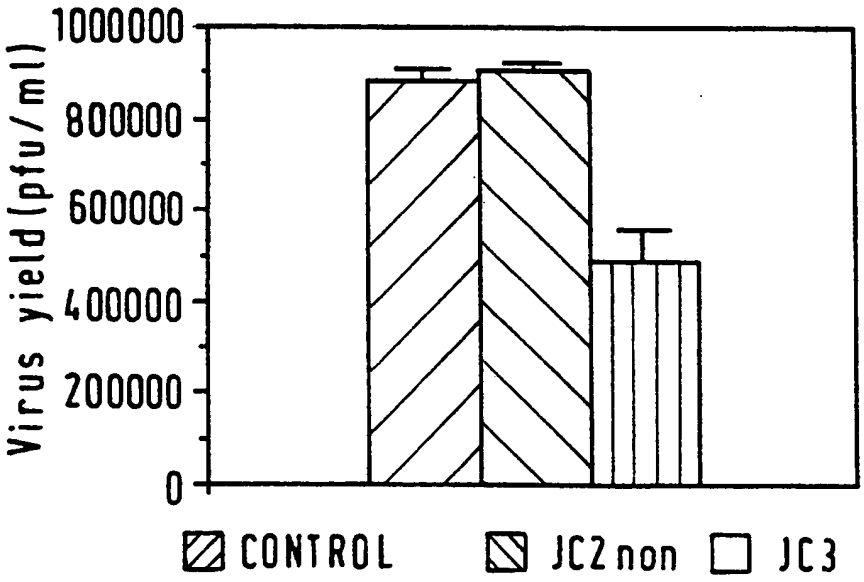

Virus yield (pfu/ml)
1000000
800000
600000
400000
200000
0
CONTROL
JC2 non
JC3

FIG. 7

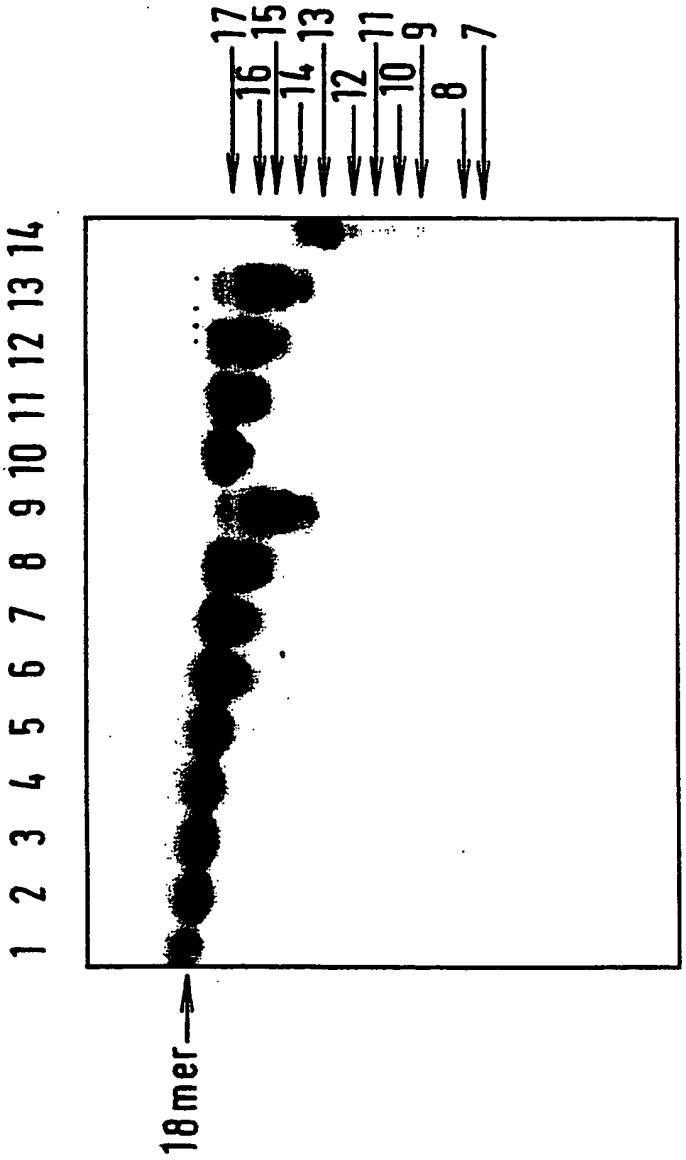
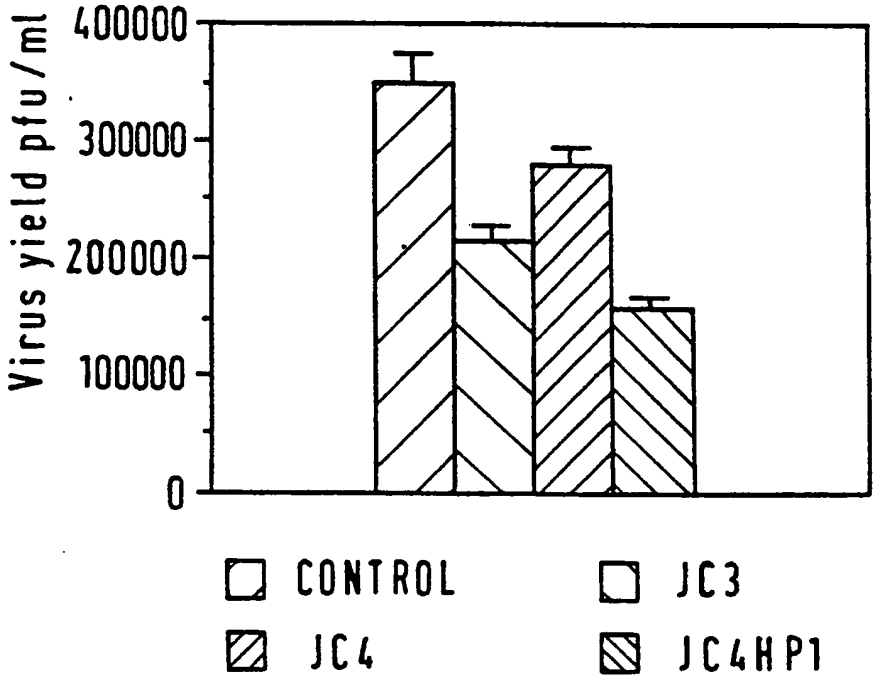


FIG. 8

JC4HP1	5'	ACCATTTCGACATCTGGA**GCGTTCGC** 3'
JC4HP2	5'	ACCATTTCGACATCTGGA**GCGAAAGC** 3'
JC4SHP2	5'	GTACCTTGACTCAGTACA**GCGAAAGC** 3'
JC4HP2S	5'	ACCATTTCGACATCTGGA**ACGAGCAG** 3'
AS3HP2	5'	TGCATAAAATGTCTGC**GCGAAAGC** 3'
JC4HP3	5'	ACCATTTCGACATCTGGA**GCGTAAGC** 3'

FIG. 14



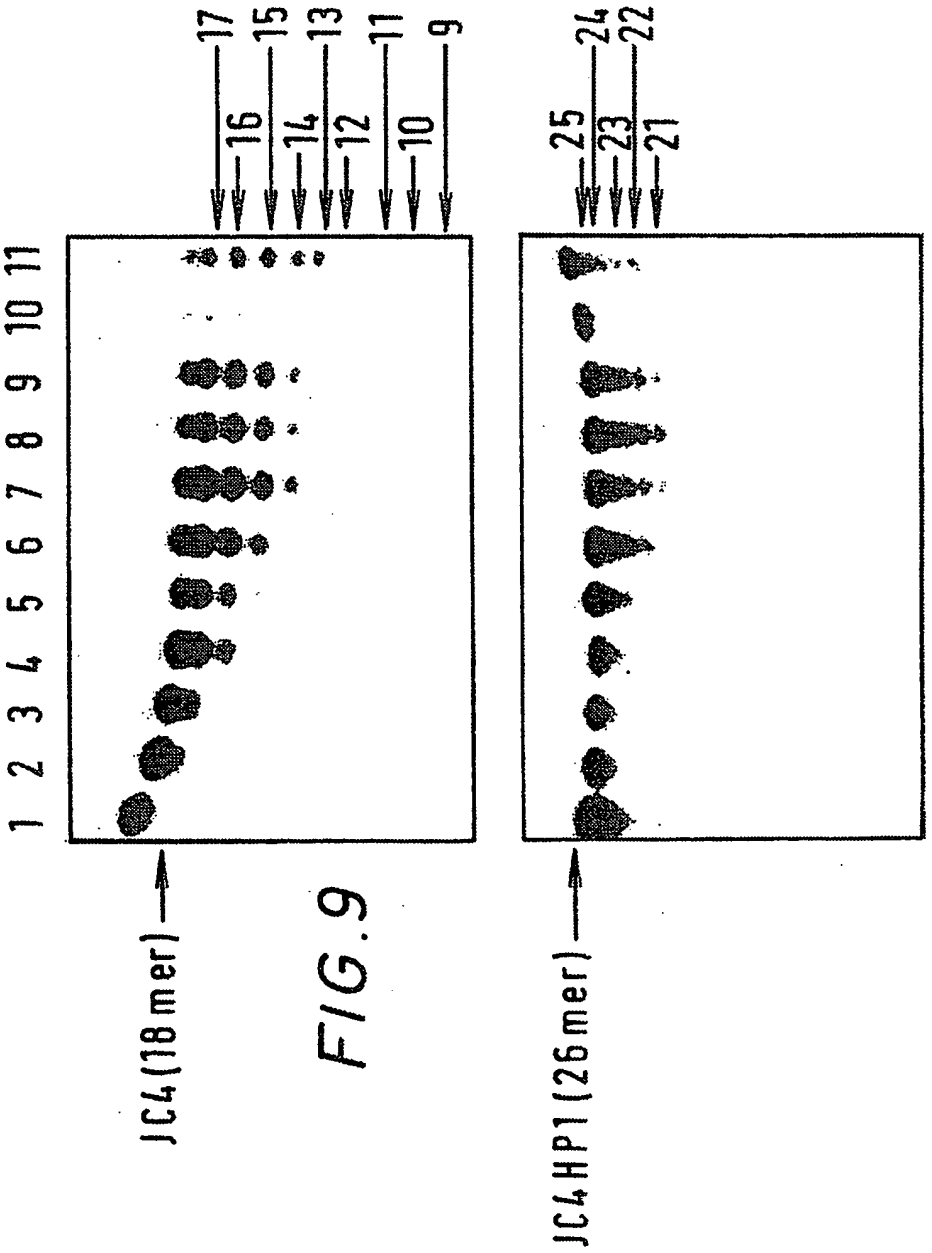

17
15
13
11
9
16
14
12
10
25
24
23
22
21
1 2 3 4 5 6 7 8 9 10 11
JC4(18mer)
JC4HP1(26mer)

FIG.9

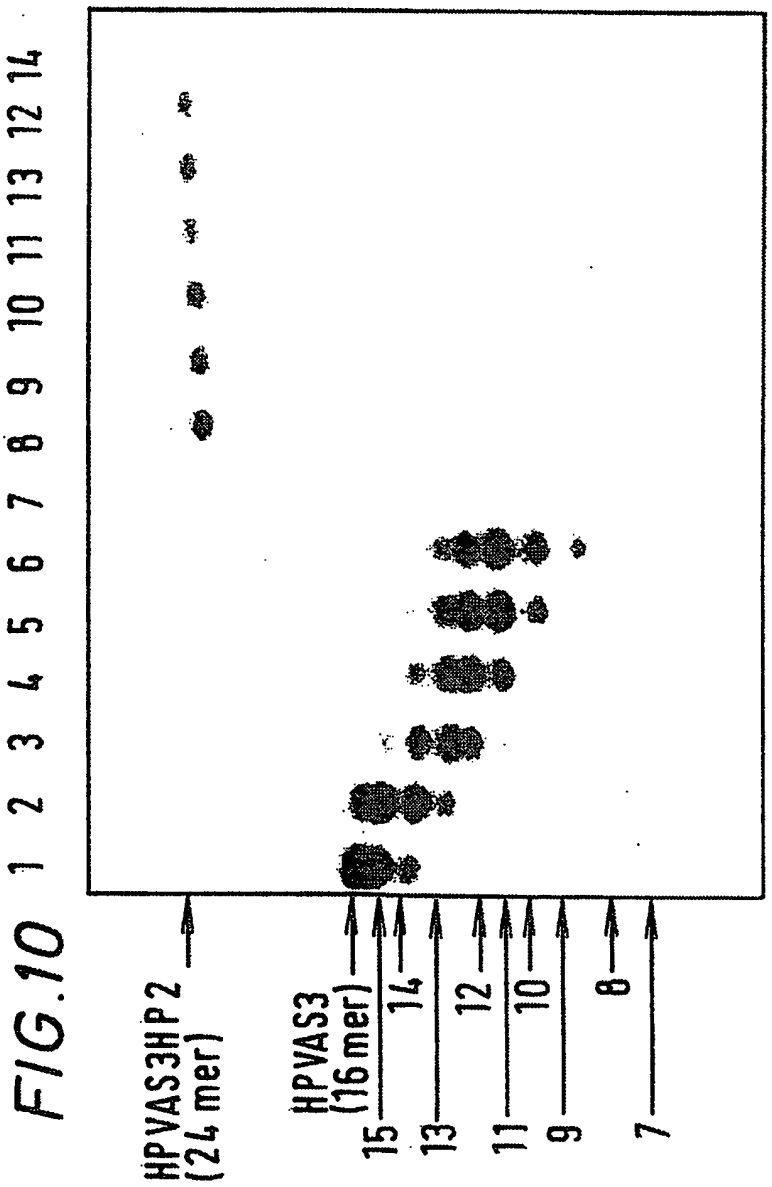

1 2 3 4 5 6 7 8 9 10 11 13 12 14
HPVAS3HP2
(24 mer)
HPVAS3
(16 mer)
15
14
13
12
11
10
9
8
7

FIG.10

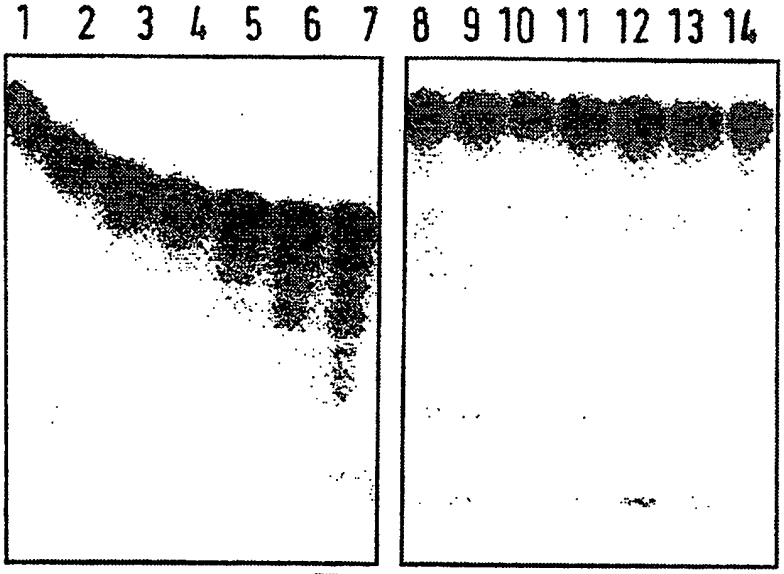


FIG. 11

FIG. 12

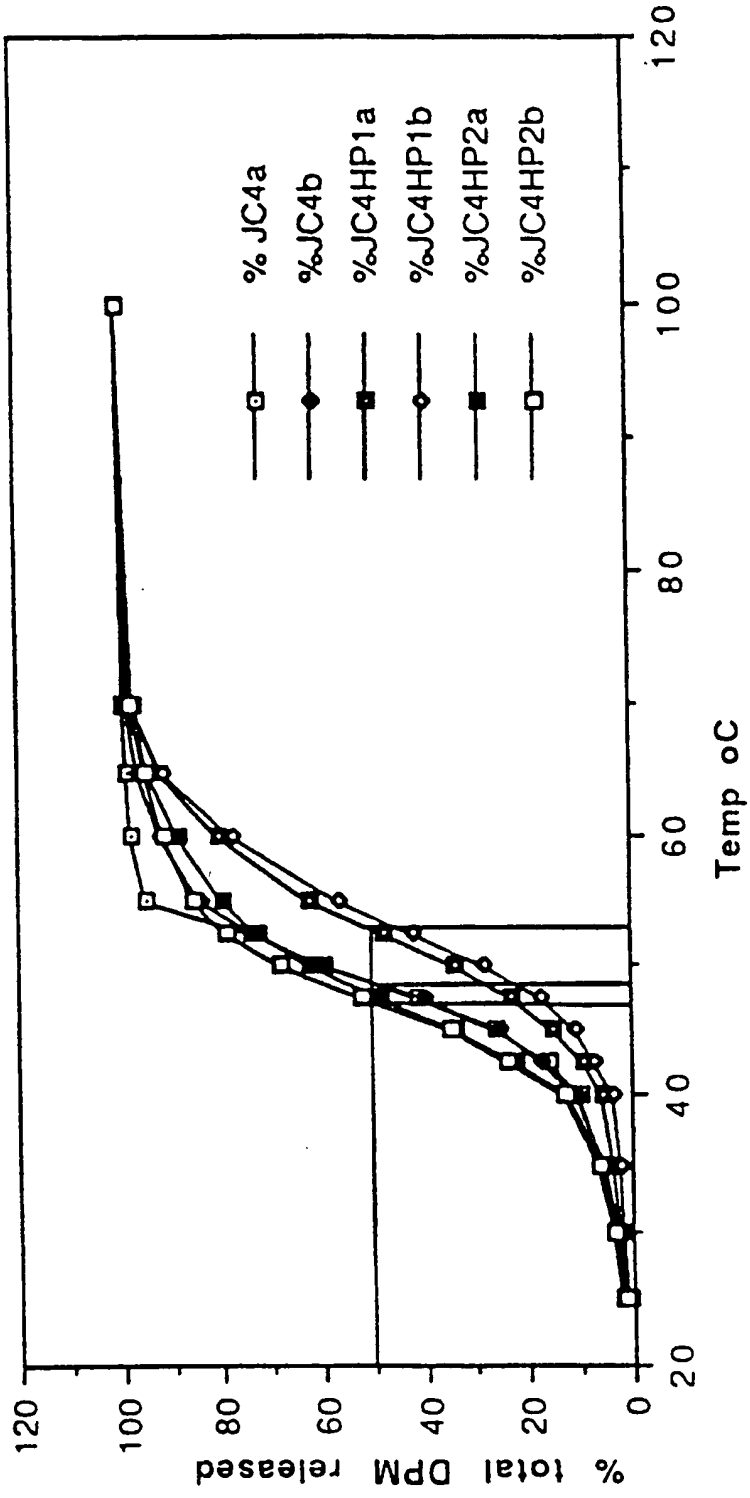
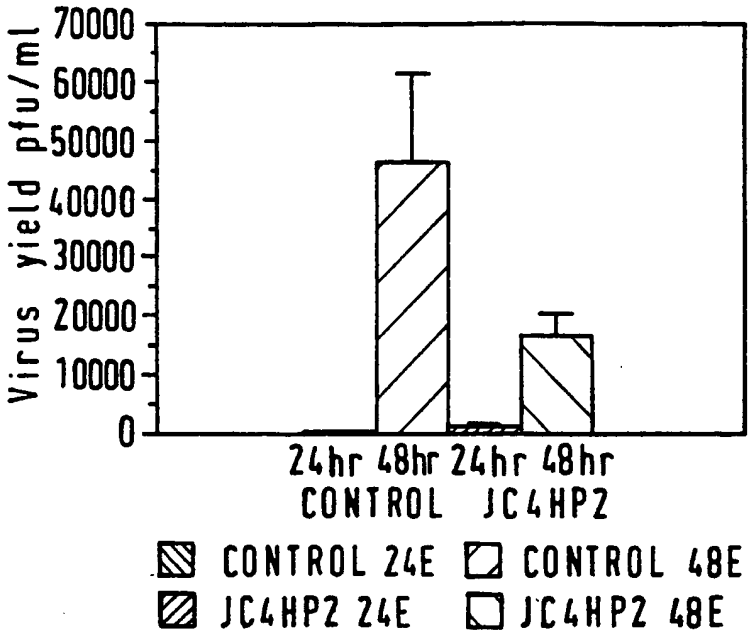

% JC4a
%JC4b
%JC4HP1a
%JC4HP1b
%JC4HP2a
%JC4HP2b
% total DPM released
Temp oC
0
20
40
60
80
100
120
20
40
60
80
100
120

FIG.13

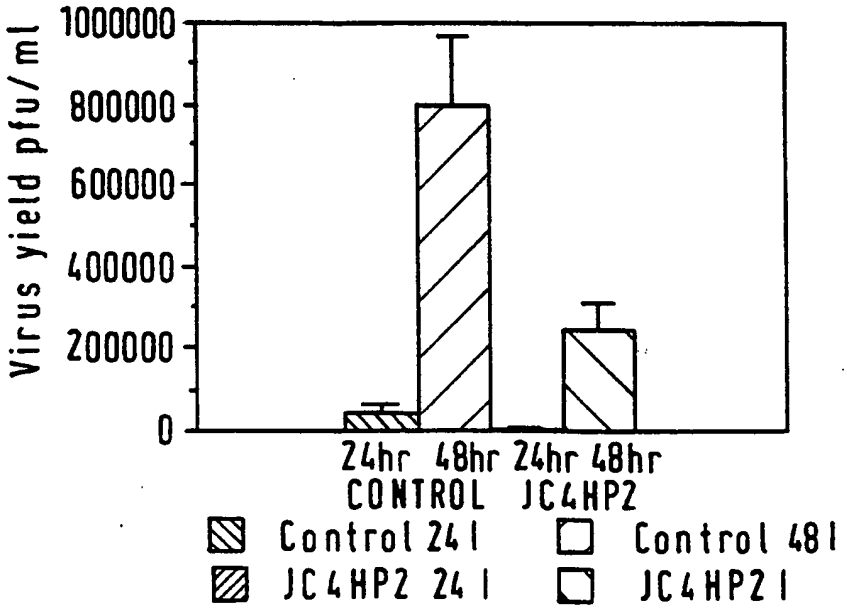
11/11

FIG. 15

(a) Extracellular virus



(b) Intracellur virus



INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 93/02409

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C12N15/11 A61K31/70 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EMBO JOURNAL vol. 8, no. 13 , 1989 , EYNSHAM, OXFORD GB pages 4297 - 4305 C. C. CASE ET AL. 'The unusual stability of the IS10 anti-sense RNA is critical for its function and is determined by the structure of its stem domain' see the whole document ---	1-5,11, 13-16
X	NUCLEIC ACIDS RESEARCH vol. 19, no. 11 , 1991 , ARLINGTON, VIRGINIA US pages 2971 - 2977 J. P. JACQUES 'Use of electrophoretic mobility to determine the secondary structure of a small antisesnse RNA' see page 2971, column 2, paragraph 1 --- -/--	1-5,11, 13-16

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
8 March 1994	27 -04- 1994

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Molina Galan, E

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INTERNATIONAL SEARCH REPORT

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO,A,92 19732 (GENSET) 12 November 1992 see abstract; claims; figures	1-5,11, 13-16
Y	EP,A,0 427 074 (MOLECULAR DIAGNOSTICS) 15 May 1991 see the whole document	1-5,11, 13-16

3

INTERNATIONAL SEARCH REPORT

...ormation on patent family members

International Application No
PCT/GB 93/02409

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9219732	12-11-92	FR-A-	2675803	30-10-92
		AU-A-	1759692	21-12-92
		CA-A-	2102229	26-10-92
		EP-A-	0581848	09-02-94
EP-A-0427074	15-05-91	AU-B-	642922	04-11-93
		AU-A-	6594990	16-05-91
		JP-A-	4008292	13-01-92